Serum Retinol Binding Protein-4 Levels in Prediabetics – Novel Biomarker of Insulin Resistance and Atherosclerosis

Ajay Chauhan, Ayushi Singhal, Parul Goyal, Anil Taneja

Abstract

Background: Atherosclerotic cardiovascular diseases are the leading cause of morbidity and mortality in both diabetics and prediabetics. In insulin resistant states, increased levels of various adipose derived cytokine (adipokine) have been found to have an important role in the process of atherosclerosis. One such novel adipokine is RBP4, (belonging to lipokalin family) which also by exerting an inflammatory process has a role in the pathogenesis of insulin resistance and CVD. Early detection of all these inflammatory cytokines may immensely help us in prognosticating the pace of disease besides instituting early interventional maneuvers.

Objective: The aim of the study was to compare serum levels of RBP4 in prediabetics and controls and to correlate levels of RBP4 with HOMA-IR and CIMT.

Methods: 60 prediabetic patients and 60 age, sex, BMI matched controls were employed in the case control study. In both cases and controls serum levels of fasting and postprandial blood glucose, glycated hemoglobin (HbA1c) and fasting insulin levels were measured. HOMA-IR values in both the groups were calculated using fasting glucose and insulin levels. Serum RBP4 levels were measured using ELISA. The values obtained were compared between cases and controls. CIMT was only measured in cases using B-mode ultrasonography.

Results: Median (IQR) of fasting plasma insulin levels (uIU/ml) in cases was 11.3 (10.175-13.505) versus that of controls which was 5.73 (4.3-7.1). HOMA-IR median (IQR) in cases and controls was 3.12 (2.73-3.595) and 1.21 (0.918-1.505) respectively. Median (IQR) for RBP4 in cases was 67.4 (46.166-111.088) which was significantly higher as compared to controls 33.92 (23.902-52.45). Significant positive correlation was seen between RBP4 with both, HOMA-IR and mean CIMT with correlation coefficients of 0.3693 and 0.621 respectively. On performing univariate linear regression analysis it was found that with increase in serum RBP4 levels by 1 mg/L, HOMA-IR and mean CIMT significantly increased by 0.007 units and 0.001 mm respectively.
Conclusion: Prediabetics have been found to have more risk of cardiovascular events as compared to normoglycemics. Early assessment of the same with the use of novel biomarkers like RBP4 can be considered for early detection of atherosclerosis in prediabetic individuals. It may further help in early intervention and thus prevention from future complications.

Introduction

Diabetes Mellitus is a chronic condition occurring due to inadequate production or inadequate action of insulin, ultimately leading to hyperglycemia. Prediabetes, regarded as a predecessor of diabetes, is a condition with elevated plasma glucose above normal levels but below that of clinical disease. According to American diabetes association it encompasses fasting plasma glucose of 100-125 mg/dl OR 2 hour postprandial blood glucose of 140-199 mg/dl OR HbA1c of 5.7-6.4%.

As per data published in international diabetic federation (IDF) diabetes atlas 2019, number of people living with impaired glucose tolerance was 25.2 million with age adjusted comparative prevalence of 3.3%.

Atherosclerotic cardiovascular diseases contribute to the world’s largest disease burden and are a leading cause of morbidity and mortality in both diabetics and prediabetics, accounting for about two third of mortality in diabetics. All components of metabolic syndrome have been found to be associated with increased risk of cardiovascular disease (CVD). In prediabetes increased risk of cardiovascular disease is multifactorial with etiologies including insulin resistance, hyperglycemia, dyslipidemia, hypertension, systemic inflammation and oxidative stress.

Risk factors leading to prediabetic state are often associated with increased expression of inflammatory cytokines and also infiltration of immune cells in adipose tissue which lead to an insulin resistance state and problems linked with this state like dyslipidemia (due to non storage of triglyceride by insulin resistant cells), hypertension, hypercoagulability and atherosclerosis. Increased levels of adipose derived cytokine (adipokine) apart from having immunological role, also have role in insulin resistant states and the process of atherosclerosis. One such novel adipokine is RBP4 (belonging to lipokalin family) which also by exerting an inflammatory process has its effect in the pathogenesis of insulin resistance and CVD.

For assessing these atherosclerotic changes in both peripheral and coronary arteries, intima-media thickness (IMT) is currently being used as a marker. Most commonly used among these is Carotid Intima-Media Thickness (CIMT), usually performed by B-mode ultrasonographic scan as it is a non invasive, inexpensive and reproducible method.

Materials and Methods

The study was conducted in the Departments of Medicine, Biochemistry and Radiology at Atal Bihari Vajpayee Institute of Medical Sciences and Dr. Ram Manohar Lohia Hospital, New Delhi.

Study Design: A Cross sectional observational study

Study Size: The study group consisted of 60 consecutive patients of prediabetes and 60 control subjects from Medicine OPD, Medicine wards and Medicine Emergencies of ABVIMS and Dr. RML Hospital, after fulfilling all inclusion and exclusion criteria and matched for age, sex and ethnicity.

Study Period: 1\(^\text{st}\) November 2018 to 31\(^{\text{st}}\) March 2020.

Calculation of Sample Size

Primary Objective

To compare serum levels of RBP4 in prediabetics and controls.

To achieve the primary objective the input for statistical sample size calculation was taken from the study by Pandey GK et al, 2015.

Patient with Impaired Glucose Tolerance showed a mean (± SD) for RBP4 of 10.5± 3.2 while those with Normal Glucose Tolerance had mean of 8.7± 2.5.

Taking these values as reference, the minimum required sample size with 90% power of study and 5% level of significance is 54 patients in each study group. To reduce margin of error, total sample size taken was 120 (60 patients per group).

Formula used was:

For comparing mean of two groups

\[ N \geq \frac{2(SD)^2 (Z_{\alpha} + Z_{\beta})^2}{(mean\ difference)^2} \]

Where \( Z_{\alpha} \) is value of \( Z \) at two sided alpha error of 5% and \( Z_{\beta} \) is value of \( Z \) at power of 90% and \( mean\ difference \) is difference in mean values of two groups.

Pooled standard deviation = \( \sqrt{(S_1^2 + S_2^2)/2} \)

Where \( S_1 \) is standard deviation of 1 group and \( S_2 \) is standard deviation of other group.

Calculations of sample size for RBP4

Pooled standard deviation = 2.87

\[ N \geq \frac{2(2.87)^2 (1.96 + 1.28)^2}{(1.8)^2} \]

\[ N \geq 53.37 \approx 54 \text{ (approx.)} \]

Inclusion Criteria

- 60 consecutive cases of Prediabetes of age 30-60 years as defined by fasting plasma glucose between 100 to 125 mg/dL OR 2-hour postprandial glucose/2-hour OGTT (after 75 gm of glucose solution ingestion) between 140 to 199 mg/dL OR HbA1c =5.7-6.4% (ADA 2016).
- 60 control subjects, matched for age, gender, ethnicity and body mass index and with fasting blood glucose of less than 100mg/dl and 2-hour postprandial glucose of less than 140 mg/dl and HbA1c less than 5.7% with no known co-morbidities as per exclusion criteria. (An informed bilingual written consent was taken from each of the patient/relatives for inclusion).

Exclusion Criteria

- Known hypertensive
- Known diabetics
- Known cases of chronic liver disease
- Known cases of non alcoholic fatty liver disease
- Known cases of myelodysplastic syndrome
- Patient on maintenance hemodialysis
- Known cases of coronary heart disease
- Known case of cerebrovascular
were: 
• Fasting plasma glucose 
• Glycated haemoglobin (HbA1c) measurement by Immuno turbidimetry method on Vitros dry chemistry analyser by NSGP guidelines.

Methods

All the cases and controls underwent following examinations and tests:

Clinical Examination

• Anthropometric measurement: The study participants were called to the Department of Medicine, Dr. RML hospital and asked to fill a pre-determined questionnaire which included baseline data about age, sex, race, ethnicity and family history of diabetes or hypertension. Then they underwent a detailed clinical examination including measurement of height (using stadiometer), weight (using a weight measurement scale) and waist circumference (using a standard measuring tape). Body Mass Index was calculated as weight in kilograms divided by height in square meters.

• Resting systolic and diastolic blood pressures were recorded twice using an automated sphygmomanometer after a 5-min rest. A mean of these two readings were taken.

Laboratory Investigations

Around 10 ml of fasting blood sample was collected after venipuncture. Samples were taken in EDTA vial for glycated haemoglobin (HbA1c) measurements. Plain (Red) vials were used to take samples for biochemical profile and separately for RBP4.

Investigations done on the patients were:
• Fasting plasma glucose 
• 2 hour postprandial plasma glucose 

Table 1: Demographic and anthropometric characteristics among cases and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cases (n = 60)</th>
<th>Controls (n = 60)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>45.68 ± 8.78</td>
<td>44.48 ± 7.44</td>
<td>0.439</td>
</tr>
<tr>
<td>Sex (%)</td>
<td>0.855</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>53.33% (n = 32)</td>
<td>51.67% (n = 31)</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (mean ± SD)</td>
<td>25.33 ± 2.65</td>
<td>24.93 ± 2.22</td>
<td>0.844</td>
</tr>
<tr>
<td>Waist Circumference (mean ± SD)</td>
<td>84.57 ± 7.3</td>
<td>82.62 ± 8.7</td>
<td>0.287</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mean ± SD)</td>
<td>116.23 ± 6.66</td>
<td>116.93 ± 8.13</td>
<td>0.541</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mean ± SD)</td>
<td>74.97 ± 5.17</td>
<td>73.43 ± 4.97</td>
<td>0.079</td>
</tr>
</tbody>
</table>

accidents (CVA) or transient ischemic attacks (TIA)

• Pregnant females
• Known cases of inflammatory bowel disease
• Known cases of Alzheimer’s disease
• Known cases of senile systemic amyloidosis
• Known smokers and alcoholics

Parameters Cases (n = 60) Controls (n = 60) P value

<table>
<thead>
<tr>
<th>Parameters (Median (IQR))</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Blood Sugar</td>
<td>110 (106-115.25)</td>
<td>86 (79-91.25)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Postprandial Blood Sugar</td>
<td>168 (156-184.25)</td>
<td>125 (117-130)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HbA1c</td>
<td>6 (5.9-6.2)</td>
<td>4.9 (4.6-5.125)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fasting Insulin Levels</td>
<td>11.3 (10.175-13.505)</td>
<td>5.73 (4.3-7.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HOMA-IR Index</td>
<td>3.12 (2.73-3.595)</td>
<td>1.21 (0.918-1.505)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum RBP4</td>
<td>67.4 (46.166-111.088)</td>
<td>33.92 (23.902-52.45)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Postprandial Blood Sugar

9. Strips were covered and incubated for 1 hour at room temperature (15-30°C) on a horizontal shaker.  
10. Again, content of each well was discarded and well was washed 5 times with 250 µl wash buffer. After final washing step, residual wash buffer was removed by firmly tapping the plate on absorbent paper.
11. Then 100 µl substrate (SUB) were added in each well.
12. Incubation was done for 10-20 minutes at room temperature in dark.
13. Finally, 100 µl stop solution

Test procedure

1. All the reagent and samples were bought to room temperature (15-30°C) and mixed well.
2. Position of standard/control/samples were marked on a protocol sheet.
3. Samples were diluted by 1:5000 in sample dilution buffer.
4. Before use, wells were washed 5 times with 250 µl wash buffer and after final washing step, residual wash buffer was removed by firmly tapping the plate on absorbent paper.
5. 100 µl of standard/control/diluted samples were added in respective wells.
6. Strips were covered and incubated for 1 hour at room temperature (15-30°C) on a horizontal shaker.
7. Content of each well was discarded and washed 5 times with 250 µl wash buffer. After final washing step, residual wash buffer was removed by firmly tapping the plate on absorbent paper.
8. 100 µl conjugate (diluted CONJ) was added in each well.
9. Strips were covered and incubated for 1 hour at room temperature (15-30°C) on a horizontal shaker.
10. Again, content of each well was discarded and well was washed 5 times with 250 µl wash buffer. After final washing step residual wash buffer was removed by firmly tapping the plate on absorbent paper.

Serum Retinol Binding Protein 4

Kits for serum retinol binding protein 4 were imported from IMMUNDIAGNOSTIK AG, Germany (Reference number K 6110). Separate kits along with buffer agents were used for cases and controls.

Principle of test

The ELISA test used was for quantitative determination of RBP/ RBP4 in plasma, urine and serum. In first incubation step, RBP/ RBP4 in samples bound to polyclonal rabbit anti RBP/ RBP4 antibodies, immobilized on microtitre plate. A peroxidase (where tetramethylbenzidine used as a peroxidase substrate) conjugated anti RBP/RBP4 antibody is used for detection and quantification. A dose response curve of absorbance unit (optical density at 450 nm) versus concentration was generated using the values obtained from standard and using this curve values of RBP/ RBP 4 were directly determined.
Ultrasonographic Examination: 

All cases underwent high-resolution B-mode ultrasonography with a 7.5 MHz linear probe, in Department of Radiology, ABVIMS and DR RML Hospital, New Delhi. CIMT was measured as distance between two echogenic lines (representing intima and media). All scans and image measurements were carried out by the same investigator, blinded to the risk factor status of the participants. The aim of the study was to assess the serum levels of Retinol binding protein 4 (RBP4) in patients with prediabetes, compare the same in normoglycemics and to correlate its levels with carotid intima media thickness (CIMT) and HOMA IR in prediabetics. It was an observational case-control study and after calculating the sample size (of 54 for RBP4) as per statistical analysis, a total of 120 patients were enrolled (60 cases and 60 controls). Matching with respect to age, sex, blood pressure and BMI was ensured. The following observation was made (Tables 1, 2, 3).

Significant difference was seen in levels of fasting plasma insulin (uIU/ml) between cases and controls (p value <0.05). Median (IQR) of fasting plasma insulin level (uIU/ml) in cases was 11.3(10.175-13.505) which was significantly higher as compared to controls where it was 5.73(4.3-7.1) (Tables 2, 4, Figure 2). Fasting plasma insulin levels (uIU/ml) were >=9 in 83.33% of cases as compared to controls where it was 16.67% of the total (Figure 3). HOMA-IR Index in our study showed median (IQR) values of 3.12(2.73-3.595) in cases and 1.86(1.22-3.12) (Tables 2, 4, Figure 2).

Results

The aim of the study was to assess the serum levels of Retinol binding protein 4 (RBP4) in patients with prediabetes, compare the same in normoglycemics and to correlate its levels with carotid intima media thickness (CIMT) and HOMA IR in prediabetics. It was an observational case-control study and after calculating the sample size (of 54 for RBP4) as per statistical analysis, a total of 120 patients were enrolled (60 cases and 60 controls). Matching with respect to age, sex, blood pressure and BMI was ensured. The following observation was made (Tables 1, 2, 3).

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was statistically significant (p value < 0.0001) (Tables 2, 5). Moreover around 91.67% of cases and 0.67 % of controls had HOMA-IR > or equal to 2 (Table 6). Median (IQR) of RBP4 level (mg/L) in cases was 67.4 (46.166-111.088) which was significantly higher as compared to control [33.92(23.902-52.45)] (p value <0.0001) (Table 2 and 7, Figure 4). Mean value of mean CIMT (mm) of study subjects was 0.61 ± 0.1 with median (IQR) of 0.6(0.5-0.7). It is shown in Table 3, Figure 1. The correlation of RBP4 and HOMA-IR Index and mean CIMT was found to be statistically significant with a correlation coefficient of 0.3693 and 0.621 respectively and p value of 0.0037 and <0.0001 respectively (Figure 5 and 6). Univariate linear regression analysis showed that with increase in levels of RBP4 by 1 mg/L, HOMA-IR, Fasting plasma insulin and mean CIMT significantly increased by 0.007 units, 0.025 uIU/ml and 0.001 mm respectively (Table 8).

**Discussion**

The study showed evidence of increased levels of RBP4 in prediabetics as compared to normoglycemics. A significant positive correlation was found between RBP4 with HOMA-IR Index (marker of insulin resistance) and mean CIMT while moderate positive correlation between RBP4 and fasting plasma insulin. It proved, the diagnostic and prognostic significance in hyperglycemia associated cardiovascular disease assessment due to alteration in the levels of the same in these states. It would thus help in early intervention to prevent any future complications.

Insulin resistance along with impairment of insulin signaling, hyperinsulinemia, and hyperglycemia by increasing glycosylation and oxidation of lipoproteins like LDL and VLDL (Very Low-Density Lipoprotein) leads to decrease in vascular compliance and also promote atherosclerosis. These vascular changes due to atherosclerosis are characterized by arterial wall lesion and endothelial dysfunction, ultimately leading to vessel wall hypertrophy which later contributes to increased risk of strokes, MI and TIA.10

One of the novel adipokines that has been found to be elevated in insulin resistant states is Retinol Binding Protein 4 (RBP4). It is secreted from liver (major fraction) and adipocytes. It carries retinol in blood from liver to peripheral tissues.11 Higher levels of RBP4 levels were found to be associated with metabolic risk factors, such as body mass Index (BMI), waist circumference, hypertension, and lipid parameters which in turn are linked with development of resistance to insulin. RBP4 itself also seems to affect the insulin signaling cascade leading to insulin resistance.12

In a prospective study done in 2014 by Ram J et al, it was found that participants who developed T2DM had higher levels of serum RBP4 as compared to non - progressors, indicating prognostic utility of RBP4 as a marker which can predict subjects who will progress from prediabetes to frank diabetes mellitus. [13] In another study by Meisinger C et al, higher levels of RBP4 was significantly associated with prediabetes, independent of other known metabolic risk factors and lifestyle variables (odds ratio 1.63 [95% CI 1.17–2.27] after multivariable adjustment).14

Kwanbunj K et al15 in their study also found a positive association of RBP4 levels with insulin resistance and thus concluded its role in stroke and
heart disease. A positive correlation between serum levels of RBP4 and CIMT (correlation coefficient of 0.623) observed in our study was correlating with other studies as well but those were done in diabetic patients only. Our study is thus the first study correlating levels of RBP4 with insulin resistance and HOMA-IR in prediabetics.

RBP4 has been found to be elevated in serum before the development of overt diabetes and it has also been correlated with components of metabolic syndrome. Various studies suggest important role of RBP4 as a direct trigger of insulin resistance and subclinical inflammation, leading to premature development of CVD and diabetes.16-18 Like these studies our study too suggests measurement of serum RBP4, a noninvasive, accessible method can be used as an early predictor for assessing the risk of CVD in prediabetic patients. It has been considered as more convenient and inexpensive test compared to vascular ultrasound for early detection and hence early intervention in vascular complications.19

### Conclusion

- In prediabetic patients levels of RBP4 may be considered as a surrogate marker for early atherosclerosis and can be used as an early predictor for the same. It may cause carotid artery atherosclerosis through the influence on insulin sensitivity, lipid metabolism, and the body oxidative stress. This molecule, when combined with other atherosclerotic markers can improve the predictive value of cardiovascular risk assessment. These patients can also be targeted for medical management with cardioprotective drugs like aspirin, statins and metformin for insulin resistance. Thus, CIMT along with RBP4, used to detect atherosclerosis in cardiovascular diseases can be employed at a much earlier stage in patients with prediabetes to estimate future CVD risk as they indicate subclinical atherosclerosis. Also RBP4 alone can be used in early detection and intervention of vascular complication as is more convenient and inexpensive compared to vascular USG.

### References


### Table 8: Univariate linear regression to find out effect of serum RBP 4 levels (mg/L) on Fasting plasma insulin level (uIU/ml)

<table>
<thead>
<tr>
<th>RBP4 (mg/L)</th>
<th>Fasting plasma insulin level (uIU/ml)</th>
<th>HOMA-IR</th>
<th>Mean CIMT (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta coefficient</td>
<td>0.025</td>
<td>0.007</td>
<td>0.0001</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.009</td>
<td>0.002</td>
<td>0.0002</td>
</tr>
<tr>
<td>Standardized coefficient</td>
<td>0.364</td>
<td>0.004</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lower bound (95%)</td>
<td>0.008</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>Upper bound (95%)</td>
<td>0.042</td>
<td>0.012</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Equation 9.838+0.025*RBP4 (mg/L) 2.662+0.007*RBP4 (mg/L) 0.49+0.001*RBP (mg/L)

Lower bound (95%) 0.008 0.002 0.001
Beta coefficient 0.025 0.007 0.0001
RBP4 (mg/L) Fasting plasma insulin level (uIU/ml) 0.49+0.001*RBP 4 (mg/L)